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FIZZ1 Stimulation of Myofibroblast Differentiation

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Bleomycin-induced pulmonary fibrosis is characterized by inflammation, emergence of myofibroblasts, and deposition of extracellular matrix. In an attempt to identify genes that may be involved in fibrosis, we used a 10,000 element (10 K) rat cDNA microarray to analyze the lung gene expression profiles in this model in the rat. Cluster analysis showed 628 genes were more than or equal to twofold up- or down-regulated, many of which were known to be involved in fibrosis. However, the most dramatic increase was observed with FIZZ1 (found in inflammatory zone; also known as RELM-α or resistin-like molecule-α), which was induced 17-fold to ~25-fold at the peak of expression. *In situ* hybridization analysis revealed FIZZ1 expression to localize primarily to alveolar and airway epithelium, which was confirmed *in vitro* by analysis of isolated type II alveolar epithelial cells. However FIZZ1 expression was not detected in isolated lung fibroblasts. Co-culture of FIZZ1-expressing type II cells with fibroblasts stimulated α-smooth muscle actin and type I collagen expression independent of transforming growth factor-β. Transfection of a FIZZ1-expressing plasmid into fibroblasts or treatment with glutathione *S*-transferase-FIZZ1 fusion protein stimulated α-smooth muscle actin and collagen I production. These results suggest a novel role for FIZZ1 in myofibroblast differentiation in pulmonary fibrosis. *(Am J Pathol 2004, 164:1315–1326)*

Pulmonary fibrosis is not an uncommon end result of a multitude of lung diseases and lung injury. Many of these result in progressive fibrosis that terminates in end-stage pulmonary disease and death from respiratory failure. Fibrosis associated with the idiopathic interstitial pneumonias is commonly an untreatable disease with significant mortality. Because the natural history of many of these diseases is unknown, animal model studies have been undertaken to fill in the gaps and to seek out clues for important pathogenic mechanisms. One of the models that have been extensively studied used bleomycin (BLM) to induce lung injury, inflammation, and fibrosis. BLM is an anti-neoplastic antibiotic, and is used clinically in treatment of various squamous cell carcinomas.[1] BLM-induced lung fibrosis is a well-characterized animal model in which a progressive fibrotic process is developed after initial lung inflammation. It is characterized by increased proliferation of fibroblasts, increased expression of cytokines such as transforming growth factor (TGF)-β1, the *de novo* appearance of myofibroblasts with their distinct α-smooth muscle actin (α-SMA)-expressing phenotype, as well as the deposition of extracellular matrix.[2–4] Accumulated evidence revealed that expression of a number of genes was elevated after BLM administration including fibrogenic cytokines such as TGF-β1 and interleukin (IL)-5, which reaches peak expression on approximately day 7 after BLM injection.[5–7] Increases in extracellular matrix expression, such as elastin, collagen I, collagen III, and α-SMA are also well established.[4,8–10] These diverse processes likely involve complex patterns of gene expression that remain to be fully elucidated. Identification of these genes and their biological activities should provide important clues to their roles in pulmonary fibrosis, and thus provide novel insights into pathogenesis and potential therapeutic approaches.

Previous studies of gene expression have provided some clues as to the potential role of certain genes in pathogenesis of fibrosis, but elucidated little in the way of uncovering the whole spectrum of possible genes that may be involved because of the limitation of standard analysis of one or a few genes. The development of oligonucleotide array or cDNA microarray technology allows the global analysis of gene expression and provides the opportunity to explore simultaneously complex patterns of gene expression in an animal model, wherein evolution from initial injury to fibrosis can be sequentially studied. Recent studies using this approach have yielded some interesting data on patterns of gene expression, in one of which a previously unsuspected role for matrilysin in BLM-induced pulmonary fibrosis is uncovered. Analysis of known genes confirmed previous evidence of early up-regulation of genes associated with inflammation followed subsequently with up-regulation of those associated with fibrosis.[11–13] A limitation is that only genes included in the microarray can be detected, nevertheless the approach has the potential of uncovering novel (or previously not suspected) genes that may play important roles in the pathogenesis of pulmonary fibrosis.

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To further examine global changes in gene expression and help identify additional, potentially novel genes that may be associated with pulmonary fibrosis, we used a 10,000 element (10 K) rat cDNA microarray to analyze the gene expression profiles in a rat model of BLM-induced pulmonary fibrosis. This approach enables screening for possible novel expressed genes and further investigation of their function. The results show more than 600 genes whose expression was significantly altered in BLM-treated lungs, some of which have been previously reported and known to be relevant to the pathogenesis of fibrosis. However, an unexpected finding was the dramatic induction of a novel molecule, previously identified as FIZZ1 (found in inflammatory zone; also known as RELM-α or resistin-like molecule-α) in BLM-induced lung injury. Initial characterization suggests expression to localize primarily to alveolar and airway epithelium, but was not expressed by fibroblasts. Studies using co-cultures of type II alveolar epithelial cells (AECs) and fibroblasts, as well as studies using a FIZZ1-expressing plasmid or rat glutathione *S*-transferase (GST)-FIZZ1 fusion protein indicated that FIZZ1 could promote myofibroblast differentiation that is independent of endogenous TGF-β1 activity. These findings suggest a novel activity of FIZZ1 that is consistent with a profibrotic role not dissimilar to that for TGF-β, at least with respect to *de novo* emergence of the myofibroblast phenotype.

Materials and Methods

Induction of Animal Model

Female-specific pathogen-free Fisher 344 rats (7 to 8 weeks old) were purchased from Charles River Breeding Laboratories, Inc. (Wilmington, MA). Pulmonary fibrosis was induced by the endotracheal injection of 7.5 U/kg body weight of BLM (Blenoxane; Mead Johnson, NJ) in sterile phosphate-buffered saline (PBS) as before.[3,4] The control group received the same volume of sterile PBS only. On days 7, 14, 21, and 28 after BLM injection, the rats were sacrificed and the lungs were removed and either immediately frozen in liquid nitrogen for mRNA analysis or used for isolation of fibroblasts and type II AECs.

cDNA Microarray

DNA microarray analysis was done essentially as previously described.[14] The sequence-verified cDNA clones on the rat cDNA microarray are available from Research Genetics (*www.resgen.com*). All chips had various control elements, which included human rat and yeast genomic DNAs, standard saline citrate, and housekeeping genes. Isolated lung tissue RNA (15 μg) was used as a template for cDNA generation using reverse transcriptase (RT) (Invitrogen, Carlsbad, CA) in the present of amino allyl-dUTP (Sigma, St. Louis, MO) that allow for subsequent fluorescent labeling of cDNA using Cy3 or Cy5 NHS ester dyes (Amersham Pharmacia Biotech Ltd., Buckinghamshire, UK). The cDNA from each time point-matched control sample was labeled with fluorescence dye Cy3 and the experimental sample with Cy5. The labeled probes were then hybridized to rat cDNA microarray at 65°C overnight. RNA samples from a total of three rats at each time point were analyzed separately by three individual chips (one for each rat sample). Thus RNA samples from different animals were not pooled before analysis. Fluorescent images were obtained using a GenePix 4000B microarray scanner (Axon Instruments, Foster City, CA).

Primary analysis was done by using the GenePix software package (Axon Instruments). Cy3 to Cy5 ratios were determined for the individual genes along with various other quality control parameters (eg, intensity over local background). The average median ratio values for all spots were normalized to 1.0. The data set was imported into Cluster Analysis of Gene Expression Dynamics (CAGED). CAGED v 1.0 software is designed to perform Bayesian model-based clustering on temporal gene expression data.[15] Briefly, CAGED considers two temporal gene expression patterns as similar when they are generated by the same unknown process, and searches for the most probable set of processes generating the observed data. Our analysis only included genes with at least one observation greater than a twofold increase or below a twofold decrease for a total of 9154 genes. We analyzed the data with an autoregressive order (Markov order) of 1, a prior precision value of 1, a γ value of 0, a Bayes factor of 1, and Euclidean distance between gene profiles to guide the analysis. The clustering method is completely described previously[15] and available at *http://www.pathology.med.umich.edu/chinnaiyan/index.html*. Clusters identified by CAGED were further analyzed using the program Cluster (log transformation of the data and average-linkage hierarchical clustering) and visualized with Tree View.[16] CAGED cluster 4, containing 648 genes, was filtered to only include genes showing a threefold difference at any two time points, and two distinct clusters of up-regulated genes were identified. CAGED cluster 4 was then filtered to only include genes showing a fourfold difference at any two time points yielding a third group of up-regulated genes.

mRNA Analysis by Real-Time Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

For quantitative mRNA analysis, total RNAs were isolated from lung tissue, fibroblasts, or type II AECs. Primer Express 2.0 software (Applied Biosystems, Foster City, CA) was used to design Taqman primers and MGB probes (6-FAM conjugated) for FIZZ1 and α-SMA, which were then purchased from Applied Biosystems (PE/ABI, Foster City, CA). The primer sequences were as follows: rat FIZZ1: forward primer, 5′CAACAGGATGAAGACTGCAACCT3′; reverse primer, 5′GGGACCATCAGCTAAAGAAG3′; and probe, 5′ 6FAM-CCCTTCTCATCTGCGTCT3′; rat α-SMA: forward primer, 5′CGGGCTTTGCTGGTGATG3′; reverse primer, 5′CCCTCGATGGATGGGAAA3′; and probe, 5′ 6FAM-TGCTCCCAGGGCTG3′.

Primers and probes for TGF-β1 and GAPDH were purchased from PE/ABI. For each assay, 100 ng of total RNA was used as template. GAPDH mRNA was used as internal control to normalize the amount of input RNA. One-step real time RT-PCR (48°C for 30 minutes, 95°C for 10 seconds followed by 45 cycles of 95°C for 10 seconds, 60°C for 1 minute) was undertaken with Taqman One Step RT-PCR Master Mix (PE/ABI) using a GeneAmp 5700 Sequence Detection System (PE/ABI).

In Situ *Hybridization*

In situ hybridization was used to localize expression of FIZZ1 in lung tissue sections. Lung tissue samples from BLM-treated and saline-treated control animals were rapidly frozen on sacrifice and 10-μm frozen sections were made. Three anti-sense and corresponding sense digoxigenin-labeled FIZZ1 oligonucleotide probes were synthesized by Integrated DNA Technologies Inc. (Coralville, CA). A mixture of three different anti-sense probes was used to increase the sensitivity of *in situ* hybridization. A mixture of the corresponding three sense oligonucleotides were used as negative control. The sequences of the three anti-sense oligonucleotide probes were as follows: 1) 5′ACGGGTAATTGGGGCGAAGAACTTTTTTAT3′; 2) 5′GACCCTACTGACGATGACCAACAAGAACAC3′; and 3) 5′GGAAGAGTAGACGCAGAAGGAAGAGGTCGA3′.

In situ hybridization was performed essentially as previously described[17] and in accordance with the kit manufacturer's instructions (Roche Molecular Biochemicals, Indianapolis, IN). Briefly, sections were digested with 1 μg/ml of Proteinase K (Invitrogen) for 30 minutes at 37°C. After postfixing by 4% paraformaldehyde (Sigma), sections were acetylated with 0.1 mol/L of triethanolamine (Sigma), and then incubated with prehybridization buffer [4× standard saline citrate containing 50% (v/v) deionized formamide (Sigma)] for 1 hour at 37°C. Sections were processed for *in situ* hybridization with mRNA *in situ* hybridization solution (DAKO, Carpinteria, CA) containing 10 ng/ml of probe mixture for 48 hours at 37°C, and then washed twice at high stringency at 37°C in 2× standard saline citrate for 15 minutes each, followed by two 15-minute washes in 0.1× standard saline citrate. After incubation with blocking buffer [100 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 0.1% Triton X-100, and 2% normal sheep serum (Sigma)], sections were incubated with anti-digoxigenin-alkaline phosphatase (1:500 in blocking buffer, Roche Molecular Biochemicals). Color was then developed with the NBT/BCIP system (Roche Molecular Biochemicals) for 2 to 24 hours followed by counterstaining with methyl green (Vector Laboratories, Inc., Burlingame, CA).

Plasmid Construction and Transient Transfection

The FIZZ1 cDNA clone used in the rat cDNA microarray chips was confirmed to be a full-length FIZZ1 cDNA by sequencing and comparing with the known sequence of rat FIZZ1. This FIZZ1 cDNA was inserted into cloning vector pT7T33D-PAC with *Eco*RI and *Not*I. The full-length rat FIZZ1 cDNA insert was next digested from pT7T3D-PAC using *Xho*I and *Hind*III, and then subcloned into pEGFP-C2 (BD Biosciences, Clontech, Palo Alto, CA) using T4 DNA ligase (Promega, Madison, WI) in accordance with the manufacturer's instructions. The identity of the construct was confirmed by sequencing.

Fibroblasts were maintained in Dulbecco's modified Eagle's medium supplemented with 10% plasma-derived fetal bovine serum, 10 ng/ml of platelet-derived growth factor, and 5 ng/ml of epidermal growth factor (R&D Systems, Minneapolis, MN). They were plated into six-well plates at a density of 2×10^5 cells/well and allowed to grow to ~80% confluence. Cells were transiently transfected by pEGFP-FIZZ1 with transfection reagent Fugene 6 (Roche Molecular Biochemicals) at a ratio of 2:3 (μg:μl), and cells were harvested after 24, 48, 72, and 96 hours, either for RNA or protein analysis. Empty plasmid pEGFP-C2 was also transfected under the same conditions and used as negative control. Where indicated transfected cells were also transfected with sense or anti-sense FIZZ1 oligonucleotides as used in *in situ* hybridization analysis, to study the effects of specific inhibition of induced FIZZ1 expression. In additional experiments FIZZ1 plasmid-transfected cells expressing FIZZ1 were sonicated and the supernates applied onto naïve (ie, not transfected) fibroblast monolayers to study the effects of FIZZ1 on these cells.

Production of Recombinant Rat FIZZ1 and Functional Study

The full-length rat FIZZ1 insert was cloned in pGEX-4T-2 (BD Biosciences, Clontech), which encodes a GST sequence. GST-FIZZ1 fusion protein was expressed in *Escherichia coli* BL21 by using bulk GST purification modules (Amersham) according to the manufacturer's instructions. Briefly, a single colony containing a recombinant pGEX-FIZZ1 plasmid was inoculated into 5 ml of 2× YTA (16 g/L tryptone, 10 g/L yeast extract, and 5 g/L NaCl) medium to grow at 37°C overnight with vigorous shaking. Then the culture was diluted into 500 ml of 2×YTA medium and grown to an $A_{600\ nm}$ of 0.6 to 0.8 (3 to ~4 hours). FIZZ1 fusion protein expression was then induced with 1 mmol/L isopropyl-β-D-thiogalactoside for an additional 2 hours. The cells were pelleted and lysed with 10 μg/ml of lysozyme followed by sonication. A 50% slurry of glutathione-Sepharose 4B was then added to the sonicates and incubated at room temperature for 30 minutes. After removing nonbound material by washing with PBS, bound protein was then eluted by addition of 10 mmol/L of reduced glutathione (in 50 mmol/L Tris-HCl). Purity of the GST-FIZZ1 fusion protein in the eluates was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. For negative control, GST was induced and purified from the parental pGEX plasmid using the same procedure.

For functional study, rat lung fibroblasts were plated onto six-well plates at a density of 2×10^5 cells/well and allowed to grow to ~80% confluence. After 24 hours of

serum deprivation, GST-FIZZ1 (4 or 8 μg/ml) or GST (8 μg/ml) was added to fibroblast monolayers. After 24 or 48 hours of incubation, the cells were harvested for analysis of α-SMA expression by Western blotting.

Cell Isolation and Co-Culture of Fibroblasts with AECs

Rat lung fibroblasts and AECs were isolated from control or BLM-treated animals, as described previously.[3,18] Isolated fibroblasts were maintained in culture and passaged as previously described.[3] Only fibroblasts between passage 3 and 5 after primary culture were used. AECs were isolated by elastase cell dispersion and IgG panning.[18] Briefly, after multiple whole lung lavages with 1 mmol/L of EGTA in balanced salt solution, porcine pancreatic elastase (4.3 U/ml; Worthington, Lakewood, NJ) was instilled via the trachea to release type II cells. Contaminating cells bearing Fc receptors were removed from the cell suspension by panning on plates coated with rat IgG (Sigma). The cells were plated onto 6-well tissue culture dishes precoated with fibronectin (R&D Systems) in Dulbecco's modified Eagle's medium supplemented with 10% newborn calf serum (Sigma). Isolated cells were evaluated by immunofluorescent staining with anti-cytokeratin5/8 (BD Biosciences, San Diego, CA), which recognizes the cytokeratins found in AEC, but not present in macrophages, fibroblasts, and endothelial cells.[18,19] After 2 days in culture, the adherent cells were consistently >90% epithelial cells. Primary cultured AECs were used without passaging. The next day after isolation, AECs (1×10^5 per well) were added onto monolayers of normal rat lung fibroblasts plated 24 hours previously into six-well plates at a density of 2×10^5 cells/well. After an additional 36 hours the cells were harvested for Western blotting analysis and enzyme-linked immunosorbent assay (ELISA) assay for quantitation of α-SMA and collagen type I expression, respectively. Where indicated, 10 μg/ml of nonimmune IgG or monoclonal anti-TGF-β1 antibody (R&D Systems) were added to the fibroblast monolayers 24 hours before adding AECs to examine the contribution of TGF-β on expression of α-SMA and collagen I. This antibody neutralizes the biological activity of TGF-β1 and TGF-β1.2 according to the manufacturer's data sheet, and the cross-reactivity with TGF-β3 and TGF-β5 was less than 2%. The cell lysates were then harvested for protein analysis 36 hours after co-culture.

ELISA Assay for Collagen Type I

This was done essentially as previously described.[20] Briefly, cell extracts (200 ng protein/well in 100 μl of PBS) were added to coat each well of a 96-well ELISA plate, and incubated at 4°C overnight. After blocking with 1% bovine serum albumin, rabbit anti-rat collagen type I (1:500; Biodesign International, Saco, ME) and polyclonal horseradish peroxidase-conjugated goat anti-rabbit IgG (BD Pharmingen) were sequentially added as primary and secondary antibodies, respectively. The color was developed with 3,3′,5,5′-tetramethylbenzidine substrates (Sigma), and read at 450 nm with an ELx 800UV universal microplate reader (Bio-Tek Instruments Inc., Winooski, VT).

Western Blotting Analysis

Western blotting to detect α-SMA protein expression was performed as previously described.[21] Ten μg of cell extract protein was separated by sodium dodecyl sulfate-polyacrylamide gel (12.5%) electrophoresis. Mouse anti-α-SMA antibody (Roche Molecular Biochemicals) and horseradish peroxidase-labeled anti-mouse IgG (Amersham) were used for immunostaining. The bands were visualized immediately by exposing to ECL Hyperfilm (Amersham), and after scanning were quantitated using 1D Image Analysis software (Kodak, Rochester, NY).

TGF-β1 Bioassay

We used a modified luciferase assay as described previously[22] to measure TGF-β1 activity. Briefly, mink lung epithelial cells transfected with plasminogen activator inhibitor-1 (PAI-1) promoter fused to the firefly luciferase reporter gene (a kind gift of Dr. DB Rifkin, New York University, New York, NY) were seeded in a 96-well plate with a density of 2×10^4 cells/well and cultured until ~80% confluent. The media were then replaced with test samples diluted (1:2 dilution) in fresh media. To measure total activity, test samples were preacidified by using Quantikine human TGF-β1 kit (R&D Systems) before assay. After a 24-hour incubation, the media were removed and the cells were lysed with reporter lysis buffer (Promega). Luciferase activity was measured by luciferase assay system (Promega) and read using a reporter microplate luminometer (Turner Designs, Sunnyvale, CA). Human TGF-β1 (R&D Systems) was used as a standard.

Statistical Analysis

All data were expressed as mean ± SE unless otherwise indicated. Differences between means of various treatment and control groups were assessed for statistical significance by analysis of variance followed by *post hoc* analysis using Scheffé's test for comparison between any two groups. A P value <0.05 was considered to indicate statistical significance.

Results

Gene Expression Pattern in BLM-Induced Lung Injury

Routine lung histopathology (hematoxylin and eosin staining) confirmed induction of the model by BLM as previously described.[3,4] Cluster analysis showed distinguishable expression patterns between normal *versus* BLM-injured lungs, with respect to distinct groups of genes involved in both the early inflammatory and sub-

sequent fibrotic responses. CAGED analysis of the temporal data set yielded five distinct clusters. The average profile of each cluster as well as the residual statistics and the expected values of an approximately normal distribution are available at *http://www.pathology.med.umich.edu/chinnaiyan/index.html*. To further examine the data, we next analyzed the results using Cluster as described in the Materials and Methods. Using Tree View, 628 of identified genes in the five clusters representing down- and up-regulated genes (on days 7, 14, 21, and 28; BLM-treated *versus* control lungs) are visualized in Figure 1.

Cluster A was composed of 38 genes whose expression was down-regulated at all time points examined. The expression of most genes in this cluster was decreased maximally at day 7 after BLM injection, and remained down-regulated up to day 28. This cluster included transcription factor nuclear factor I/B, CD36 (an adhesion receptor for collagen and thrombospondin), and anti-inflammatory molecule CC10 (Clara cell 10-kd protein). Cluster B contained 26 genes that were up-regulated and peaking at day 7, with gradual decline toward control baseline levels at day 28. It included inflammation/fibrosis-related genes, such as phospholipase A2, complement component 1, procollagen C-proteinase enhancer, CTGF (connective tissue growth factor), C/EBPδ (CCAAT enhancer binding protein δ), gelatinase A/MMP-2 (matrix metalloproteinase-2), and TIMP-1 (tissue inhibitor of metalloproteinase-1). Although TGF-β1 expression was also elevated, the increase was only 1.5-fold and thus did not meet the cutoff of twofold increase for inclusion in this panel (data not shown). In cluster C a group of 33 genes were more gradually up-regulated, peaking closer to day 21 followed by a subsequent decline. In this group are such fibrosis-related genes as procollagen I and other genes that encode for proteins involved in extracellular matrix metabolism, such as MMP-14 and stathmin (microtubule disassembly molecule). The largest cluster D contained more than 200 genes that were coordinately expressed at a very late stage of BLM-induced lung fibrosis. Many of these were genes that encode for proteins related to extracellular matrix deposition/turnover, such as procollagen I, lamin B, preprocathepsin D, and zyxin (a local adhesion protein). Interestingly, TTF-1 (thyroid transcription factor) whose overexpression is associated with pulmonary fibrosis,[23] was detected in this group of up-regulated genes. Cluster E was the smallest with nine genes whose expression pattern displayed rapid up-regulation by day 7 after BLM administration, and continued to increase up to day 21. Some of the genes in this group such as FIZZ1, phospholipase D2, and neogenin, had not been associated previously with BLM-induced lung fibrosis. Among these genes, FIZZ1 was the most distinctive in terms of the magnitude of increase. It was increased eightfold at day 7, 17-fold at day 14, 25-fold at day 21, and remained 14-fold higher than control at day 28 (Figure 2). No other gene examined exhibited such a level of increase as a result of BLM-induced lung injury and fibrosis.

Consistent with the significant role of TGF-β1 in BLM-induced lung fibrosis, in all five differentially expressed clusters, we found a batch of TGF-β1-inducible genes including C/EBPδ, MMP-2, MMP-14, tyrosine sulfotransferase, CBP/p300-interacting transactivator, and serine protease inhibitor. cDNA microarray analysis of TGF-β1-treated rat lung fibroblasts also showed significant induction of several components in cluster E, such as phospholipase D2, apolipoprotein E4, neogenin, and chimerin by TGF-β1 (data not shown).

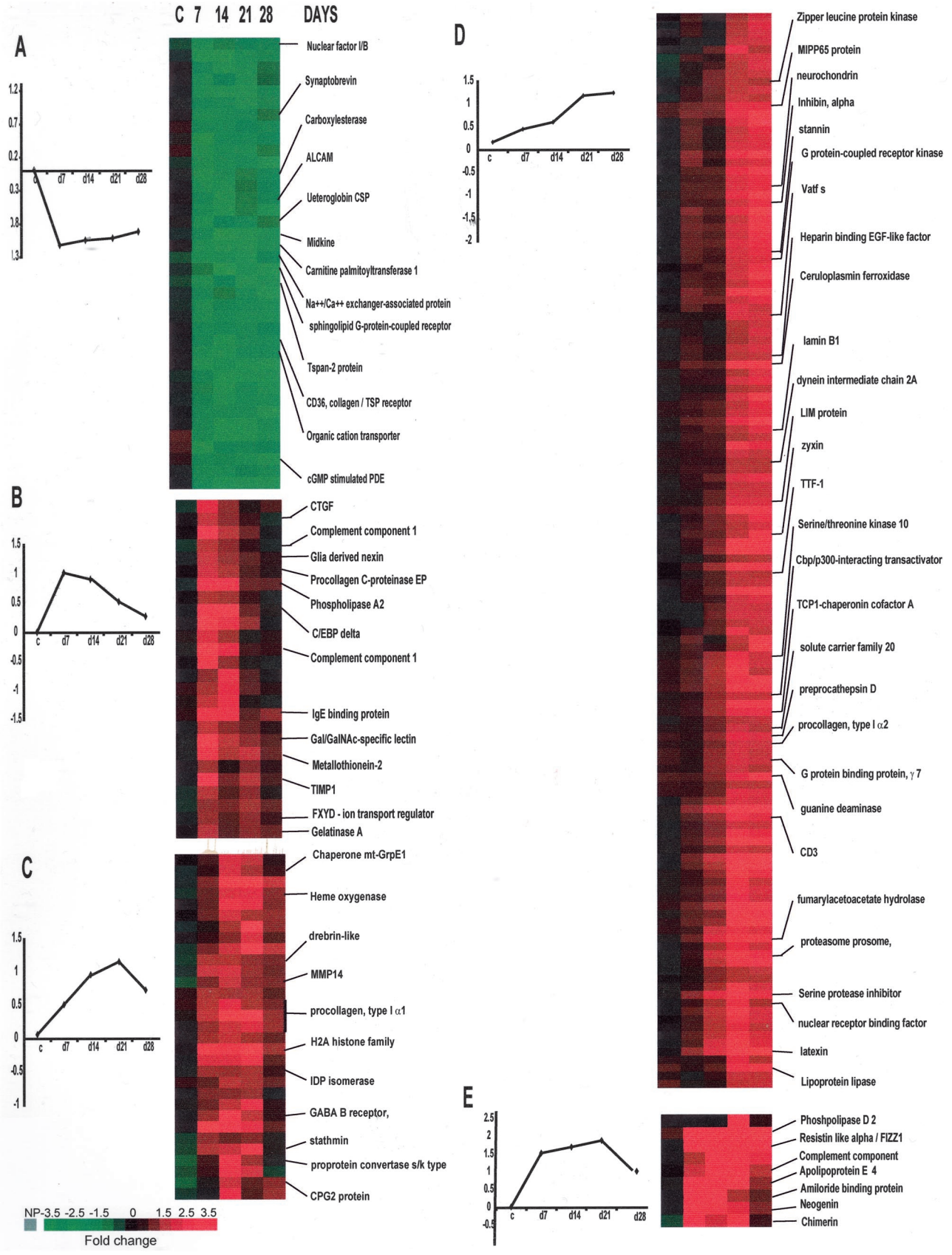
FIZZ1 mRNA Expression Was Induced in Injured Lung

To validate the cDNA microarray data, we performed real-time RT-PCR analysis on lung tissue RNA samples from control and BLM-injured lungs from the various time points. The results showed that FIZZ1 mRNA expression in control lung tissue was at very low levels at all time points examined (Figure 3). However in BLM-injured lungs, FIZZ1 mRNA expression was rapidly induced to more than 80-fold control levels as early as day 7 after BLM treatment. This level of increase was sustained for at least another week, before dropping to >20-fold increase over controls on day 21, and was still >10-fold increased by day 28. Clearly the microarray analysis underestimated the level of induction of FIZZ1, especially at the days 7 and 14 time points. This time course of FIZZ1 expression paralleled the peaks of α-SMA and collagen I expression (data not shown) as previously reported for this model.[4]

To determine the *in situ* localization and cellular sources of induced FIZZ1 gene expression in BLM-induced pulmonary fibrosis, *in situ* hybridization analysis for FIZZ1 mRNA was undertaken in lung tissue from day 7 BLM-treated rats. The results show that FIZZ1 mRNA was localized to cells lining recognizable airways as well as alveolar spaces, consistent with expression in airway and AECs (Figure 4). Some of the latter have the cuboidal morphology consistent with AECs. Additionally, a number of scattered FIZZ1-positive mononuclear cells resembling monocytes/macrophages were observed. Because these *in situ* hybridization results and previous reports[24] suggest the lung epithelial cells are possible sources of FIZZ1 gene expression. AECs were isolated from control and day 7 BLM-treated rats to see if they express induced FIZZ1 gene. Using real-time RT-PCR, low levels of FIZZ1 mRNA were detected in AECs from control rats, which was induced more than 10-fold in cells from day 7 BLM-treated rats. Interestingly, this marked induction in FIZZ1 expression was not accompanied by a significant change in TGF-β1 expression (Table 1). In contrast to AECs, FIZZ1 expression could not be detected by RT-PCR in lung fibroblasts isolated from both control and BLM-treated rats.

Effects of FIZZ1-Expressing AECs on Fibroblasts

Because AECs were shown to be a major source for FIZZ1 during BLM-induced lung injury, their effects on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H2A histone family
IDP isomerase
GABA B receptor,
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Ceruloplasmin ferroxidase
lamin B1
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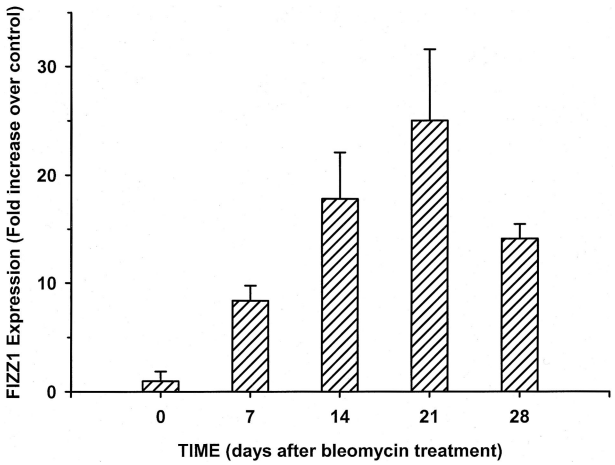

Figure 2. Kinetics of FIZZ1 induction from microarray data. The results from microarray analysis for FIZZ1 were expressed as fold increase over control lung tissue, and plotted as a function of time after BLM treatment. Means ± SE of triplicate animals are shown.

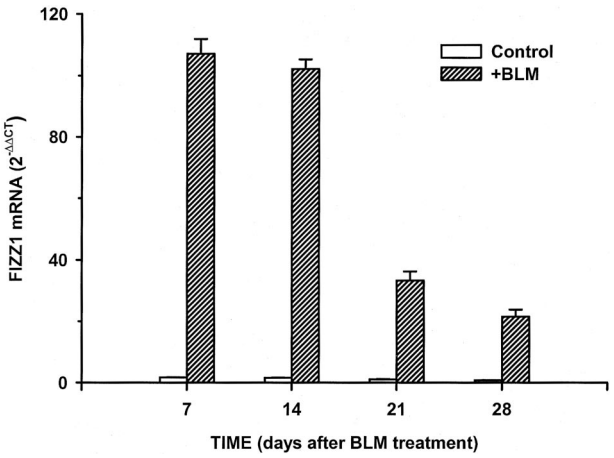

Figure 3. Kinetics of FIZZ1 mRNA expression by real-time RT-PCR. Lung tissue total RNAs were isolated after BLM or saline injection at the indicated time points, and subjected to Taqman real-time RT-PCR. GAPDH signals were used as internal controls. Results are shown as the threshold cycle (CT) at which an increase of reporter fluorescence (ΔRn) can first be detected. Amounts of FIZZ1 mRNA were normalized to GAPDH signals and expressed as $2^{-}\Delta\Delta CT$. Means ± SE of triplicate samples are shown. All mean values in the BLM-treated groups were statistically different ($P < 0.001$) from their corresponding control (saline-treated) groups.

certain fibroblast functions related to fibrosis could be used to explore the potential role of this molecule in fibrosis. AECs from control and day 7 BLM-treated rats were added onto normal rat lung fibroblasts and after 36 hours of co-culture, the cells were harvested for analysis of type I collagen and α-SMA protein expression, two parameters of fibroblast activation and myofibroblast differentiation. Because these are not expressed by AECs, the analysis of total cell lysates should only report on expression by the fibroblasts in the co-culture. The results revealed that fibroblast type I collagen expression was significantly increased by co-culture with AECs from BLM-treated animals, and only slightly increased by co-culture with control AECs, which was not statistically significant by ELISA assay (Figure 5A). Control AECs were unable to stimulate fibroblast α-SMA expression, whereas AECs from BLM-treated animals caused a more than twofold increase by Western blotting analysis (Figure 5a). As expected, TGF-β1 significantly stimulated type I collagen and α-SMA expression in fibroblasts. Lung fibroblasts from BLM-treated animals exhibited higher levels of α-SMA expression relative to those from control animals as before,[4] which were further increased in response to co-culture with AECs from BLM-treated rats (Figure 5B). However the fold increase induced by these AECs was significantly greater for the control fibroblasts *versus* that in fibroblasts from BLM-treated rats despite the higher absolute values in the latter.

Because FIZZ1 expression in AECs from BLM-treated rats was significantly higher than that in control AECs (Table 1), these results suggest that the differential effects of the two AECs on these fibroblast parameters may be because of FIZZ1. Because TGF-β1 expression was comparable between AECs from control and BLM-treated animals (Table 1), the observed differential effects on fibroblast endpoints were unlikely to be because of this cytokine. This was confirmed by co-culture studies in the presence of neutralizing antibodies to TGF-β1 (Table 2). Addition of 10 μg/ml of these antibodies caused 60% inhibition of the TGF-β activity in culture supernates (data not shown), but failed to have an effect on α-SMA or collagen type I expression (Table 2). Although this makes it unlikely that TGF-β is the cause for the effects on α-SMA and collagen type I expression, the role of other factors secreted by AECs cannot be ruled out.

Effects of FIZZ1 Expression and GST-FIZZ1 Fusion Protein on Fibroblasts

The AEC co-culture studies suggest the ability of FIZZ1 to activate fibroblasts, in terms of extracellular matrix production and myofibroblast differentiation. To confirm such a role for FIZZ1, a rat FIZZ1-expressing plasmid (pEGFP-FIZZ1) was created for transfection studies to see if forced FIZZ1 expression could activate fibroblasts, which were shown to lack the ability to express FIZZ1. Transfection efficiency was monitored by assessing the expression of EGFP (green fluorescence) by fluorescence microscopy. As early as 24 hours after transfection, significant numbers of cells exhibited green fluorescence in their cytoplasm. The number of EGFP-positive cells reached a peak level of ~30% at 72 hours and

Figure 1. Gene expression pattern (signature) of BLM-induced pulmonary fibrosis. Gene expression profiles of control and BLM-treated rat lung were analyzed by cDNA microarray analysis. Each experimental sample was hybridized against its matched control RNA obtained at the indicated time points (days). Expression data of 10,000 genes were analyzed by Cluster Analysis of Gene Expression Dynamics (CAGED) v 1.0 software. CAGED analysis revealed five distinct clusters, **A** to **E**. Each **row** represents a single gene and each **column** an average of the data from individual arrays of samples from three individual animals, at any given time point. Thirty-eight of the down-regulated genes are shown in **cluster A**. The up-regulated genes were separated into four groups: **cluster B**, consisting of 26 genes that were up-regulated early, **clusters C** and **E**, with 33 and 9 genes, respectively, that increased in the middle period of fibrosis. The late up-regulated genes displayed in **cluster D** were arrived at by applying a filter of any two time points showing fourfold difference on the 522 genes identified by CAGED analysis.

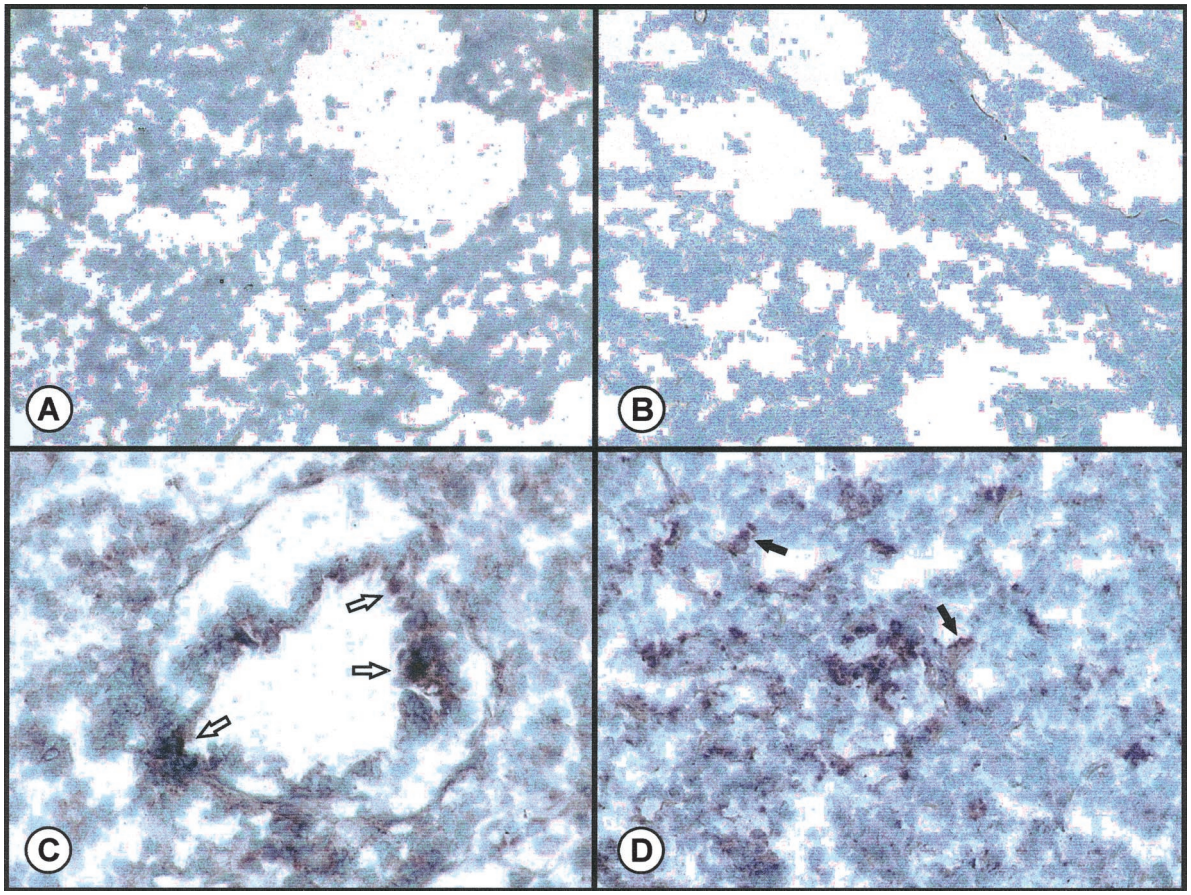

Figure 4. Localization of lung FIZZ1 expression by *in situ* hybridization. Lung tissue sections from a day 7 BLM (**A**, **C**, and **D**) or saline-treated (**B**) rat were analyzed by *in situ* hybridization for FIZZ1 mRNA expression. Digoxigenin-labeled sense (**A**) and anti-sense (**B–D**) oligonucleotides against FIZZ1 were used as probes. The positive signals (purple) were localized to airway (**C**, **open arrows**) and alveolar (**D**, **solid arrows**) epithelia in day 7 BLM-treated rat lung, but were essentially undetectable in control saline-treated lung (**B**). No signal was observed in day 7 BLM-treated lung section hybridized with the control sense probes (**A**). Original magnifications, ×400.

began to decline by 96 hours (data not known). FIZZ1 expression in the transfected cells was confirmed using real-time RT-PCR. Expression was detectable as early as 4 hours after transfection with pEGFP-FIZZ1, and gradually increased up to 24 hours (Figure 6A). FIZZ1 mRNA was undetectable in untreated fibroblasts or those transfected with the empty vector control (data not shown).

The functional effects of this forced FIZZ1 expression on fibroblasts were then examined. Transfection with the FIZZ1 plasmid caused a significant increase in type I collagen expression at both 48 and 72 hours after transfection relative to that of cells transfected with the empty vector (Figure 6B) or of untreated cells (data not shown). Constitutive expression of collagen by these rat lung fibroblasts was not significantly affected by transfection with the empty vector. Effects on α-SMA expression was evaluated by real-time RT-PCR and showed significant induction of its expression by the FIZZ1 expression vector above that by the empty vector (Figure 6C), which was not significantly different from that in untreated cells (data not shown). Thus forced FIZZ1 expression in fibroblasts stimulated collagen production and myofibroblast differentiation. Because TGF-β1 is known to stimulate fibroblast matrix production and induce myofibroblast differentiation as assessed by α-SMA expression, the possibility that FIZZ1 may promote these endpoints by

Table 1. Expression of FIZZ1 and TGF-β1 in Lung Cells

In vivo treatment	Cell type	mRNA species	
		FIZZ1	TGF-β1
Saline	AEC	1.2405 ± 0.043	0.9982 ± 0.0721
BLM	AEC	14.2352 ± 4.0903*	1.2206 ± 0.0310
Saline	Fibroblast	Undetectable	Not done
BLM	Fibroblast	Undetectable	Not done

FIZZ1 and TGF-β1 mRNA abundance (expressed as $2^{-\Delta\Delta CT}$, and shown as means ± SE of triplicate samples) was measured by real-time PCR in AECs and fibroblasts isolated from day 7 saline or BLM-treated animals.
*$P < 0.05$ for BLM-treated *versus* saline-treated controls.

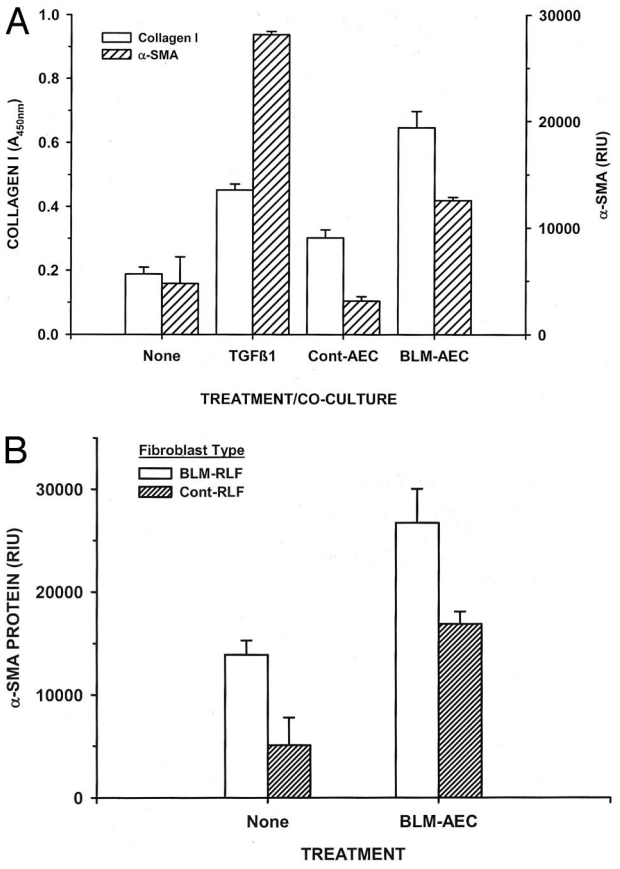

Figure 5. Effects of FIZZ1-expressing AECs on fibroblasts. FIZZ1-expressing AECs from control (Cont-AEC) or day 7 BLM-treated (BLM-AEC) rats were co-cultured with normal rat lung fibroblasts (Cont-RLF) or day 7 BLM-treated (BLM-RLF) as described in Materials and Methods. After 36 hours of incubation cell extracts were prepared for measurement of type I collagen by ELISA (right *y* axis in **A**), and of α-SMA by Western blotting (left *y* axis in **A** and ordinate in **B**). The Western blotting data were expressed as relative integration units (RIU) after measurement of net intensity of scanned bands from Western blots (in both **A** and **B**). TGF-β1 was added to fibroblasts as a positive control. Means ± SE of triplicate samples are shown. The stimulatory effects of BLM-AEC on both parameters were significantly different ($P < 0.05$) from their corresponding values for Cont-AEC and untreated fibroblasts. TGF-β1 effects were significantly higher than untreated cells. Cont-AEC did not have significant effects on fibroblasts. In **B** the effects of BLM-AEC co-culture on α-SMA expression in Cont-RLF were compared to those in BLM-RLF by Western blotting analysis. Both Cont-RLF and BLM-RLF expressed significantly higher α-SMA when co-cultured with BLM-AEC, and in each instance the expression in BLM-RLF was significantly higher than that in Cont-RLF. Results from triplicate samples were shown and expressed as in **A**.

stimulating endogenous TGF-β1 expression was examined. The results show that forced FIZZ1 expression in fibroblasts failed to significantly affect TGF-β1 expression, both at the mRNA or protein levels (data not shown). Thus the effects of FIZZ1 on collagen and α-SMA expression were independent of any significant alteration in endogenous TGF-β1 expression.

To confirm that the observed effects were specifically because of FIZZ1 expression, cells transfected with the FIZZ1 expression plasmid were treated with anti-sense or sense FIZZ1 oligonucleotides. Transfection with the FIZZ1 plasmid induced α-SMA expression, which was inhibited by the anti-sense but not by the sense oligonucleotides (Figure 7a). This demonstrated that the effects noted in transfected fibroblasts were because of FIZZ1 expression. To further establish that the noted effects

Table 2. Effects of Anti-TGF-β1 Antibody on AEC-Induced Fibroblast α-SMA and Collagen Type I Expression

Treatment	Protein (% of control)	
	α-SMA	Collagen I
IgG	145.2 ± 9.65	185.2 ± 3.70
Anti-TGF-β1 antibody	143.2 ± 3.39	184.5 ± 2.80

To rule out a role for TGF-β1, BLM-AECs were co-cultured with fibroblasts in the presence of neutralizing anti-TGF-β1 antibody or nonimmune IgG, and the effects on α-SMA (Western blotting) and collagen type I (ELISA) expression examined. Results were expressed as a percentage of untreated fibroblasts (without AEC co-culture) and the means ± SE (triplicate samples) are shown. The antibody failed to affect the stimulatory effects of co-culture with BLM-AECs.

were indeed because of FIZZ1, sonicates of fibroblasts transfected with the FIZZ1 plasmid were tested for their ability to induce myofibroblast differentiation in control nontransfected fibroblasts. When such extracts were incubated with the latter cells, α-SMA expression was stimulated relative to those treated with extracts of cells transfected with the empty vector (Figure 7b). This strongly suggested a specific effect by FIZZ1 on myofibroblast differentiation. For further confirmation, recombinant rat GST-FIZZ1 fusion protein was produced and purified. Treatment of fibroblasts with this fusion protein, but not GST, stimulated α-SMA expression to levels comparable to the effect of TGF-β1 (Figure 7c). This provided conclusive proof that FIZZ1 could stimulate myofibroblast differentiation.

Discussion

Pulmonary fibrosis is often a progressive disorder that culminates in respiratory failure and a fatal outcome. Despite recent advances in current understanding of certain aspects of the fibrotic process, much remains to be discovered with respect to key pathogenic mechanisms. Current evidence using microarray approaches suggests that altered expression of a complex set of genes may be involved in this disease process, although the specific pathogenic role(s) of many of the affected genes has not been fully elucidated.[11,12] In this study, cDNA microarray analysis was used to evaluate the spectrum of gene expression that may be altered in BLM-induced lung injury and fibrosis.

Consistent with previous studies,[11,12] the results showed alterations in expression of many genes that are known to be involved in pulmonary fibrosis, such as interstitial procollagen genes. Additionally, there were significant alterations in the expression of inflammation-related genes, including cytokines and chemokines. Interestingly, among the down-regulated genes in BLM-injured lungs was CC-10, which is known to inhibit the production of IL-1 and tumor necrosis factor-α.[25] It is also notable that transgenic mice overexpressing IL-4 in the airways have decreased expression of CC-10.[25,26] Another down-regulated gene was CD36, which may be involved in activation of TGF-β and whose expression is known to be reduced by TGF-β1 in human macrophages.[27] Two members of the MMP family, MMP-2/

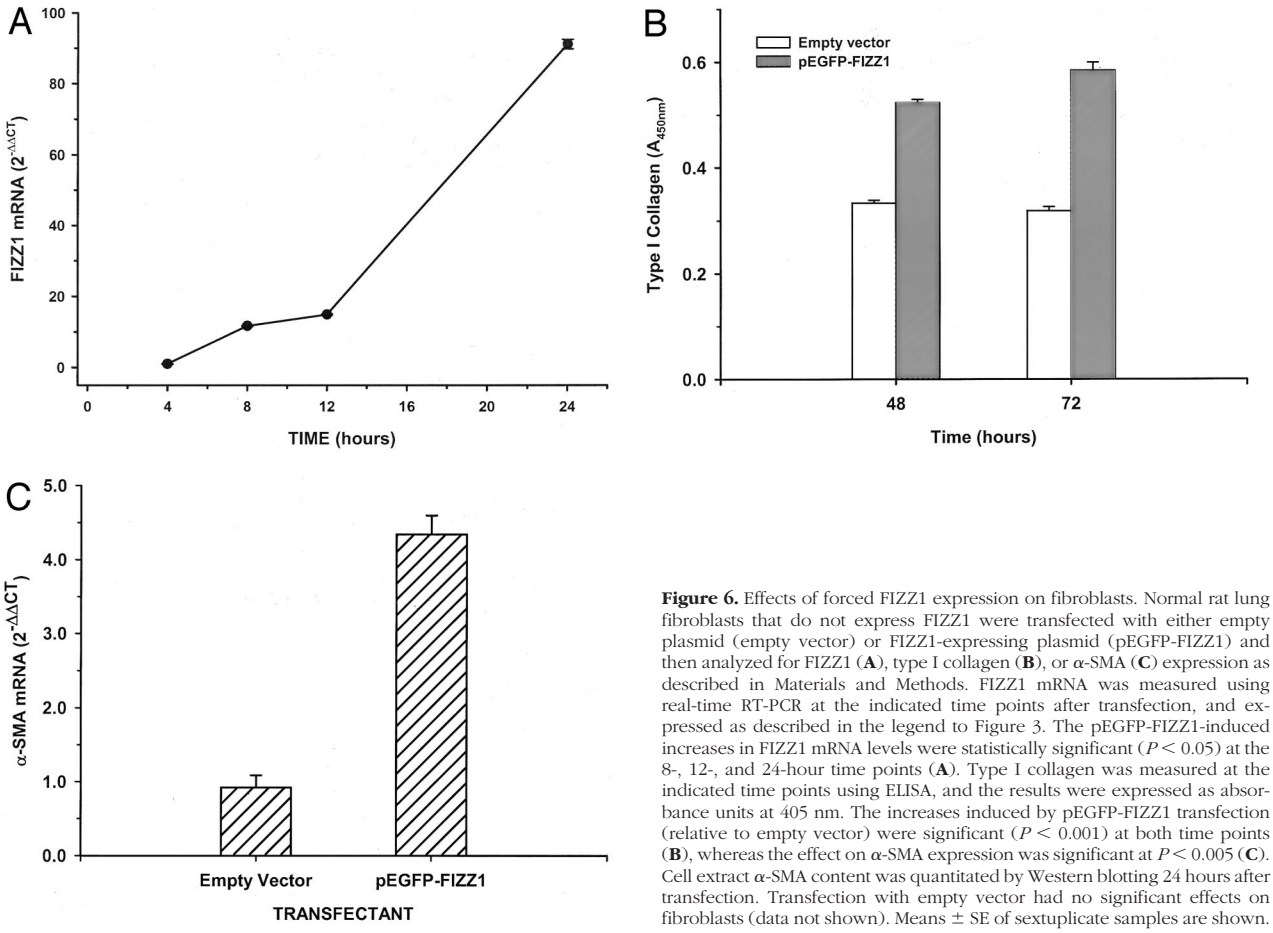

Figure 6. Effects of forced FIZZ1 expression on fibroblasts. Normal rat lung fibroblasts that do not express FIZZ1 were transfected with either empty plasmid (empty vector) or FIZZ1-expressing plasmid (pEGFP-FIZZ1) and then analyzed for FIZZ1 (**A**), type I collagen (**B**), or α-SMA (**C**) expression as described in Materials and Methods. FIZZ1 mRNA was measured using real-time RT-PCR at the indicated time points after transfection, and expressed as described in the legend to Figure 3. The pEGFP-FIZZ1-induced increases in FIZZ1 mRNA levels were statistically significant ($P < 0.05$) at the 8-, 12-, and 24-hour time points (**A**). Type I collagen was measured at the indicated time points using ELISA, and the results were expressed as absorbance units at 405 nm. The increases induced by pEGFP-FIZZ1 transfection (relative to empty vector) were significant ($P < 0.001$) at both time points (**B**), whereas the effect on α-SMA expression was significant at $P < 0.005$ (**C**). Cell extract α-SMA content was quantitated by Western blotting 24 hours after transfection. Transfection with empty vector had no significant effects on fibroblasts (data not shown). Means ± SE of sextuplicate samples are shown.

gelatinase A and MMP-14 showed increased expression up to as long as day 21 after BLM administration. This is consistent with recent microarray studies showing that MMP-2, MMP-7 (matrilysin), and MMP-14 were increased in fibrotic lung from human UIP (usual interstitial pneumonia) patients as well as from fibrotic murine lungs.[11,12] Furthermore MMP-7 knockout mice were shown to be protected from BLM-induced fibrosis,[11] suggesting an important role for at least this member of the MMP family.

Examination of gene expression changes that occur at later time points after BLM treatment revealed induction of CBP/p300 and TTF-1, which may be of potential importance in lung injury and fibrosis. As a transcriptional co-activator, CBP/p300 is involved in competitive antagonism between TGF-β1 and interferon-γ regulation of procollagen I gene expression by interaction with Smad3 and Stat-1α.[28] TTF-1 is a critical regulator of transcription for surfactant protein, and its transgenic overexpression in the lung causes disruption of alveolar septation, lung inflammation, and fibrosis associated with eosinophil infiltration, and increased expression of eotaxin and IL-6.[23]

A novel and unexpected finding of this microarray analysis was the identification of FIZZ1 and neogenin as the most highly induced genes in BLM-injured lungs. Neogenin, a DCC (deleted in colorectal carcinoma)-related gene is known to be induced by TGF-β in dermal fibroblasts,[29] but its function in the context of tissue injury, repair, and fibrosis is unknown. Induction of FIZZ1 was even higher than that for neogenin, and its dramatic induction as early as day 7 (>20-fold) after BLM treatment was sustained or persisted up to as late as day 28 (>10-fold). This induction of FIZZ1 expression from microarray analysis was confirmed by real-time RT-PCR, which revealed an even greater induction of FIZZ1 mRNA (>30-fold). In a model of ovalbumin-induced allergic pulmonary inflammation, its expression was markedly increased and localized primarily to the bronchial and alveolar epithelia.[24] Consistent with this finding in an airway inflammation model, highly induced FIZZ1 gene expression also localized to the airway epithelium and AECs in BLM-injured lungs as assessed by *in situ* hybridization. Furthermore this cell-type-specific expression was confirmed in isolated AECs from BLM-injured lungs, but could not be detected in isolated lung fibroblasts from control or injured lungs. FIZZ1 gene expression in AECs from BLM-injured lungs was 100-fold higher than that in control AECs, whereas TGF-β1 expression was not significantly different between these two cell populations of AECs.

FIZZ1 (also known as RELM-α) is a recently described protein in rodents, and belongs to a novel class of cysteine-rich secreted proteins known as the FIZZ/RELM (resistin-like molecules) family.[24,30,31] The FIZZ family has a unique tissue expression pattern. FIZZ1, initially found in lung allergic inflammation, is expressed predominantly in white adipose tissue with lower levels of expres-

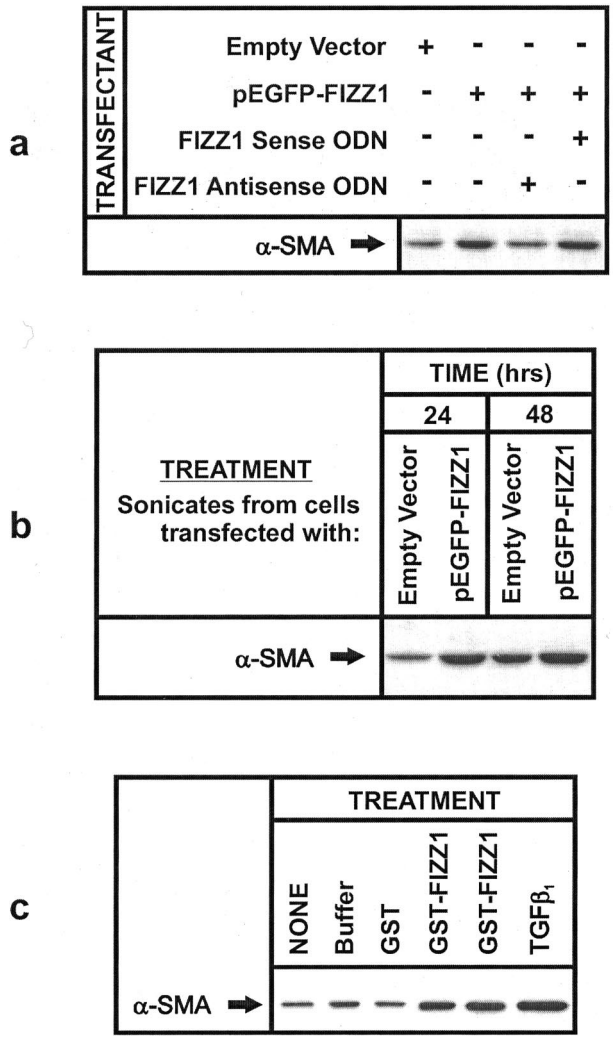

Figure 7. Effects of FIZZ1 expression and GST-FIZZ1 fusion protein on fibroblasts. FIZZ1 expression plasmid (pEGFP-FIZZ1) was transfected into fibroblasts and the effects of sense and anti-sense FIZZ1 oligonucleotides were examined. FIZZ1 plasmid-transfected cells showed heightened α-SMA expression by Western blotting analysis, which was inhibited by anti-sense FIZZ1 oligonucleotides but not by sense oligonucleotides (**a**). When cell sonicates from FIZZ1 plasmid-transfected cells were added onto nontransfected fibroblasts, enhanced α-SMA expression was noted by Western blotting analysis relative to fibroblasts treated with sonicates from cells transfected with the empty vector at both 24 and 48 hours of incubation. (**b**). Finally, addition of GST-FIZZ1 fusion protein to fibroblasts stimulated α-SMA expression by Western blotting analysis, whereas GST had no significant effects. TGF-β1 was used as positive control (**c**). Representative results from three independent experiments are shown in each panel.

sion noted in lung, heart, and mammary glands. FIZZ2 is expressed only in the gastrointestinal tract, particularly the colon, but not in white adipose tissue where FIZZ3 is exclusively expressed.[24] Although human FIZZ2 and FIZZ3 have been reported, there is as yet no known human homologue of FIZZ1.[30] The FIZZ family of proteins exhibit high homology between human (only FIZZ2 and FIZZ3), mouse and rat species. The cDNA of murine FIZZ1 (mFIZZ1) with 536 bp has been isolated and cloned from normal mouse lung. Its sequence encodes 111 amino acids with the predicted molecular weight of 9.4 kd. The N-terminal contains a signal peptide (amino acids 1 to 23) cleavable between amino acids 23 and 24. The C-terminal has a highly conserved, cysteine-rich domain in which the spacing of all 10 cysteine residues is invariant in the FIZZ/RELM family.[24,30] FIZZ1 lacks a cysteine residue in the highly variable N-terminus, which is present in both FIZZ2 and FIZZ3 in multiple species. This particular cysteine residue is necessary for homodimerization that is observed with FIZZ2 and FIZZ3, but not with FIZZ1, which is secreted as a monomer.[32] In addition, FIZZ1 is able to form hetero-oligomers with FIZZ3 but not FIZZ2.[33] These structural studies indicate that FIZZ1 is a secreted protein and likely to be a signaling molecule. This is supported by a study showing that FIZZ1 is secreted into the culture medium after transfection of FIZZ1 into 293T human embryonic kidney cells.[30] However there is some uncertainty with respect to the actual biological function or activity of this family of molecules. An early study reveals that recombinant FIZZ1 inhibits *in vitro* the nerve growth factor-mediated survival of rat embryonic dorsal root ganglion neurons, as well as nerve growth factor-induced expression of calcitonin gene-related peptide in these neurons.[24] Another study indicates that FIZZ1 is able to inhibit the differentiation of 3T3-L1 preadipocytes into adipocytes without affecting cell proliferation.[33] The significance of these reported activities of FIZZ1 vis-à-vis the pathogenesis of pulmonary fibrosis is unclear. Specifically, despite its expression in inflamed airways and now in fibrotic lung, the role of FIZZ1 in inflammation and/or fibrosis, if any, is unknown.

To begin to address this issue, the effects of co-culturing FIZZ1-expressing AECs on fibroblasts were examined. AECs from BLM-treated rats were used initially as a source of FIZZ1. These studies show that control AECs that expressed very low levels of FIZZ1 did not significantly activate isolated normal rat lung fibroblasts with respect to type I collagen and α-SMA expression. However high FIZZ-1-expressing AECs from BLM-treated rats significantly stimulated both type I collagen and α-SMA expression in fibroblasts. Both of these endpoints are reflective of fibroblast activation and differentiation to myofibroblasts, whose *de novo* induction and persistence are thought to be important in the pathogenesis of pulmonary fibrosis.[5] Although TGF-β1 is a known inducer of this activated fibroblast phenotype,[34,35] the noted differential effects of these two AEC populations on fibroblasts appeared not to be mediated by this cytokine because TGF-β1 expression was not significantly different between AECs from BLM-treated *versus* control animals. Moreover, neutralizing anti-TGF-β antibodies failed to affect this activity on fibroblasts. Nevertheless the role of other or additional AEC-derived mediators could not be ruled out.

Thus to confirm that the effects of AECs on fibroblasts were because of FIZZ1, a FIZZ1-expressing plasmid, pEGFP-FIZZ1, was constructed using the whole length cDNA used in the microarray chip. Transfection of this FIZZ1 plasmid into normal rat lung fibroblasts stimulated the expression of both type I collagen and α-SMA, which was not observed when cells were transfected with the empty vector. The effect on α-SMA expression by the FIZZ1 plasmid was blunted by treatment with anti-sense but not sense FIZZ1 oligonucleotides, thus confirming that the noted effect was specifically because of FIZZ1. Furthermore, sonicates of fibroblasts transfected with the

FIZZ1 plasmid, but not the empty vector, were able to stimulate α-SMA expression. These effects on fibroblasts were not accompanied with a significant change in TGF-β1 expression, thus confirming that FIZZ1 could activate fibroblast and myofibroblast differentiation independent of TGF-β1. Conclusive proof was obtained by use of GST-FIZZ1 fusion protein, which was shown to directly stimulate α-SMA expression. These results taken together suggest a novel and key function or activity for FIZZ1 that has a direct bearing on the pathogenesis of pulmonary fibrosis. Further studies are necessary to confirm the importance of such an activity *in vivo*, and to understand regulation of FIZZ1 expression in pulmonary fibrosis.

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